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Review

Signaling through C2 domains: More than one lipid target ☆

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ABSTRACT

C2 domains are membrane-binding modules that share a common overall fold: a single compact Greek-key motif organized as an eight-stranded anti-parallel β-sandwich consisting of a pair of four-stranded β-sheets. A myriad of studies have demonstrated that in spite of sharing the common structural β-sandwich core, slight variations in the residues located in the interconnecting loops confer C2 domains with functional abilities to respond to different Ca^{2+} concentrations and lipids, and to signal through protein–protein interactions as well. This review summarizes the main structural and functional findings on Ca^{2+} and lipid interactions by C2 domains, including the discovery of the phosphoinositide-binding site located in the β3–β4 strands. The wide variety of functions, together with the different Ca^{2+} and lipid affinities of these domains, converts this superfamily into a crucial player in many functions in the cell and more to be discovered. This Article is Part of a Special Issue Entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

1. Introduction

C2-domains are independently folded modules, of about 130 residues, found in a large and diverse set of eukaryotic proteins [1–3]. They were discovered as the second of the four conserved domains in classical PKCs (α, βI, βII, γ) responsible for Ca^{2+}-dependent membrane binding [4–6]. Novel PKCs (ε, η, δ, θ) lack the Ca^{2+}-dependency of the classical isoforms but soon it was described that they contain a V0/C2 region at their N-terminal [7] that interacted with negatively charged phospholipids [8–13]. A wide variety of proteins containing C2-domains have been identified from their discovery, most of them are involved in membrane trafficking and fusion, and in signal transduction (Fig. 1).

These domains are structurally defined as all-beta protein members of the C2-domain superfamily of Ca^{2+}/lipid-binding domains (CaLB) (Structural Classification of Proteins, SCOP: http://scop.mrc-lmb.cam.ac.uk/scop/ and Class Architecture Topology Homology, CATH: http://www.cathdb.info/). SCOP classification is based on two circularly permuted topologies that render a different orientation of the eight β-strands to each group of domains [14]. Having one or the other topology is not a determinant factor for the domain's function, including the Ca^{2+}-binding properties (Fig. 2). The superfamily includes two families: (i) the PLC-like variants, also known as the P-family or the type II topology and (ii) the synaptotagmin-like variants also referred to as the S-family or the type I topology (Table 1).

Recent update of the CATH database collects 127 domains in this superfamily, with 102 unique PDB entries classified in 3 structural and 80 functional family clusters [15]. Additional bioinformatics analysis, based on sequence profile searches, phylogenetic and phyletic-pattern and structure-prediction, have implemented the long list of proteins containing C2 domains. This information has been included in the PFAM (Protein Families) database (http://pfam.sanger.ac.uk) and

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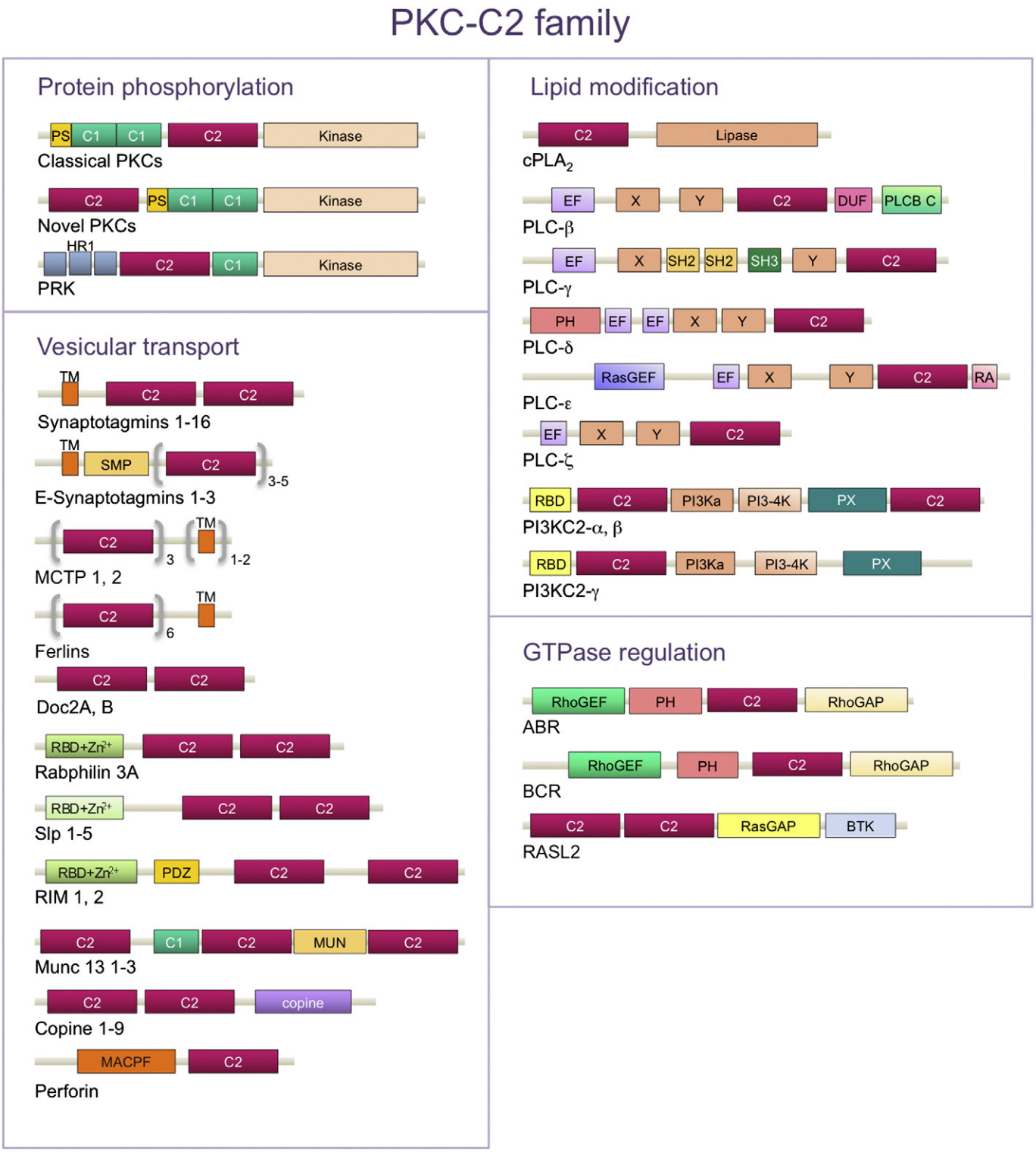

Fig. 1. Domain structures of proteins included in the PFAM/PKC-C2 family. The proteins have been classified into four groups depending on their functions: protein phosphorylation, vesicular transport, lipid modification and GTPase regulation. Parentheses in the diagram are associated with the number of C2 domains in a protein. Abbreviations: PS, pseudosubstrate; PKC, protein kinase C; HR1, protein kinase C-related kinase homology region 1; TM, transmembrane region; SMP, synaptotagmin-like-mitochondrial-lipid binding protein; MCTP, multiple C2 domain and transmembrane region proteins; DOC2, double C2-like domain-containing protein; Slp, synaptotagmin-like protein; RIM, Rab3-interacting molecule; cPLA2, cytosolic phospholipase A2; PLC, phospholipase C; EF, EF-hand motif; DUF, domain of unknown function; SH2, Src homology 2; SH3, Src homology 3; PH, pleckstrin homology domain; GEF, guanine nucleotide exchange factor; RA, ras association domain; RBD, ras binding domain; PX, P40/47phox homology domain; GAP, GTPase-activating protein; BTK, Bruton's tyrosine kinase.

provides a wider view about the evolution, structure and function of the members of this superfamily (Table 2) [16–18]. Interestingly, these studies show evidences that the last eukaryotic common ancestor contains about 9 families that participate in many functions related to membranes like repair and vesicular trafficking, actin and tubulin anchoring to the plasma and vesicular membranes, localization of small GTPases to membranes, lipid-based signal transduction and ciliogenesis [17,19]. This classification also shows that the calcium-dependent membrane interaction is a derived feature restricted to the PKC-C2 family, an ability that was acquired by its last common ancestor.

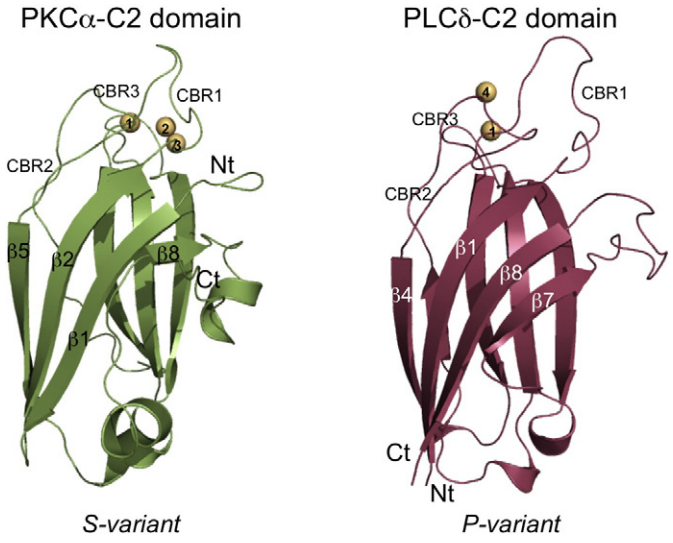

Fig. 2. SCOP classification of the C2 domain superfamily. Cartoon of the C2 domain structures representative of topology I, PKCα (PDB: 1DSY) and topology II, PLCδ (PDB: 1DJI). They are depicted in green and raspberry, respectively. Bound Ca^{2+} ions are shown as yellow spheres and the localization of the three calcium binding regions (CBR1-3) are marked. Note that due to the circular permutation, the β1 strand in PKCα overlaps with β8 strand in PLCδ, leaving the N- and C-terminals near the top of the domain in the first case and at the bottom in the second.

This property is not retained by all members of the family, and some of them have lost the Ca^{2+}-binding residues but still bind lipids, i.e., C2 domains of novel PKCs [17].

In this review, we will focus on describing the basic structural and functional properties of C2 domains belonging to the PFAM/PKC-C2 family (Fig. 1 and Table 2), and notably the new findings obtained in the last years. These domains are considered peripheral proteins that are water-soluble and associate reversibly with lipid bilayers. They represent a form of membrane docking for soluble proteins (e.g., classical PKCs, PLCδ or $cPLA_2$) or additional membrane docking for integral proteins (e.g., synaptotagmins). Their main role is to act as Ca^{2+}-activated membrane modules [2,3,20]. The molecular anatomy of the calcium-binding region will be described taking into account the structural information available to date and will be compared with two singular examples (piccolo and rabphilin 3A) that deviate from the basic structure. We will also overview their different lipid selectivity at the calcium-binding region and will include the recent reports that demonstrate that some of these domains are also able to specifically interact with PtdIns(4,5)P_2 through a different motif located in the β3–β4 strands [21]. This implies a new scenario in the field of conditional peripheral proteins based on the possibility of dual target-binding to the same domain.

Table 1
SCOP classification of the C2 domain superfamily (Calcium/Lipid-binding domain).

PLC-like variants ***P-family*** ***Topology type II***	***Synaptotagmin-like variants*** ***S-family*** ***Topology type I***
PI-specific phospholipase C isoenzyme D1 (PLC-D1)	Synaptotagmin 1-C2A/C2B
Cytosolic phospholipase A (cPLA)	Synaptotagmin 3-C2A/C2B
PTEN tumor suppressor	Synaptotagmin 4-C2A
Phosphoinositide 3-kinase gamma	Regulating synaptic membrane exocytosis protein, RIM2-C2A
PKC delta	Piccolo-C2A
PKC epsilon	PKC alpha
E3 ubiquitin-protein ligase Itchy	PKC beta
Unc-13 homolog A	Rabphilin 3A-C2A/C2B
Multiple C2 and transmembrane domain-containing protein 2 (MCTP2)	C2 domain protein At1g63220
Fantom (RPGR-interacting protein 1-like protein	Synaptotagmin 13-C2A
Phospholipase C-beta-2	Synaptotagmin-like protein 4-C2A
E3 ubiquitin-protein ligase NEDD4[a]	B/K protein-C2A
PKC etha	PKC gamma
	Synaptotagmin VII-C2A/C2B

[a]Proteins included in the shaded cells correspond to structures included in the PDB (Protein Data Bank) after the last update of the SCOP database.

2. Molecular anatomy of the calcium binding region

Pioneering structural studies of the C2 domains of C2A-synaptotagmin 1, PLCδ1, PKCβ and α, and $cPLA_2$ [22–26] demonstrated that this region is formed by three Ca^{2+}-binding loops (CBR1-3) located on one side of the domain. Five highly conserved aspartate residues coordinate two or three Ca^{2+} ions in most cases (Fig. 2). Taking the C2 domain of PKCα as an example, there are three aspartate residues (D187, D246 and D248) that form a pseudo-dyad symmetry axis and their carboxylate groups contribute to the coordination spheres of Ca1 and Ca2 (Fig. 3A). D193 at the CBR1 and D254 at the CBR3 contribute an oxygen ligand to the coordination sphere of Ca1 and Ca2, respectively. Additional residues in the region also contribute to Ca^{2+} coordination, for example the main chain oxygen of M186 and W247 [25]. PKCα-C2 domain also binds a third Ca^{2+} that is coordinated by oxygens from the main chain of R252, side chain of T251 and carboxylate group of D254 (Fig. 3A) [27].

Structural overlap of the C2 domains of PKCα with PKCβ and C2A-synaptotagmin 1 showed that they were very similar (r.m.s. deviation between equivalent Cα atoms was 0.43 Å and 0.91 Å, respectively) [22,25,26], all of them bind 2–3 calcium ions and share conserved residues that contribute to Ca^{2+} coordination in the same way (Fig. 3B).

The C2 domain of PLCδ is a topological variant of synaptotagmin 1 [28,29] with its CBR located on the same end of the β-sandwich. It also binds two calcium ions: one of them is common to synaptotagmin 1 (Ca1), the second one locates closer to the CBR1 and needs contribution from N677 in the CBR2 (Ca4) (Fig. 3C). Substitution of calcium by lanthanum in crystallization experiments indicated that the domain could also bind a third calcium ion in position Ca2 like C2A-synaptotagmin 1 (Fig. 3C) giving a general idea that both calcium-binding modes were similar and no big rearrangements in the domain occurs upon metal binding [29]. The existence of three Ca^{2+}-binding sites in the domain was further confirmed by a combination of site directed mutagenesis and ITC experiments [30,31].

The structure of the C2 domain of $cPLA_2$ was solved soon after those of PLCδ and synaptotagmin 1. Its overall structure revealed a topology like PLCδ with a similar distribution of the CBRs. CBR2 and CBR3 exhibit main chain conformations almost identical to the conformations observed for the other C2 domains (Fig. 3C and D). CBR1 is longer than the other two and has a turn of α-helix that confers a greater rigidity to this region [24,32]. Only two Ca^{2+} are located in positions Ca1 and Ca4 [24], the presence of N95 instead of an aspartate residue impedes the bipartite coordination with a third calcium ion (Fig. 3D).

Comparison of the Ca^{2+}-bound and free forms of C2A-synaptotagmin 1, PLCδ1 and PKCβ and α demonstrated that very subtle rotations of some side chains occur upon Ca^{2+} binding without big conformational changes of the backbone [22,25,28,33] together with a higher structural stabilization [22,28,34–38]. Considering all these properties, it was difficult to figure out how calcium binding was modulating the C2 domain function without producing a conformational change. Observations that Ca^{2+}-binding induces a switch that changes the electrostatic potential of the CBR suggested that this might be a key event to regulate the interactions with membranes and other proteins [39,40]. This hypothesis was extended when the crystal structure of the C2 domain of PKCα was solved in complex with Ca^{2+} and phosphatidylserine [25] and provided an additional explanation now that Ca^{2+} appeared acting as a bridge connecting the protein and the lipids in the membrane as they fill the incomplete coordination sphere of the Ca^{2+} bound to the domain.

Table 2
Classification of the C2 domain clan (PFAM Database).

Family	Proteins/functions
PKC-C2 (PFAM: PF00168)	The majority of these domains bind to membranes in a Ca^{2+}-dependent manner. It includes classical and novel PKCs, copines, ferlins, perforin, ubiquitin ligases, tollip, phospholipases A, C and D, the synaptotagmin family and proteins related to them like extended synaptotagmins, synaptotagmin-like, rabphilin, DOC2, RIM, copines and UNC13 among others.
PI3K-C2 (PFAM: PF00792)	Protein associated with the kinase domain in phosphatidylinositol-3 and phosphatidylinositol-4 kinases.
PTEN-C2 (PFAM: PF10409)	Associated to phosphatases and many other uncharacterized proteins.
AIDA-C2 (PFAM PF14186)	Found in Aida protein and related to proteins containing cytoskeletal interacting domains.
B9-C2 (PFAM PF07162)	Found in ciliary basal body associated proteins like MKS1, Xbx-7 and Stumpy/Tza-1.
DOCK-C2 (PFAM PF14429)	Associated with Dock180/Dock1 and Zizimin proteins, which are atypical GTP/GDP exchange factors for the small GTPases Rac and Cdc42, related to cell-migration and phagocytosis.
NT-C2 (PFAM PF10358)	Identified in several microfilament/actin and endocytosis related proteins like EEIG1, Sym-3 and EHBP1.
CEP76-C2 (PFAM PF15627)	C2 domain contained in centrosomal protein p76, CEP76. It is involved in regulation of centriole duplication.
CC2D2AN-C2 (PFAM PF15625)	First C2 domain of ciliary CC2D2A protein that is involved in ciliogenesis and several ciliopathies. They are predicted to mediate membrane localizations for Y-shaped linkers of transition zone of cilia.

The Ca^{2+}-binding modes surveyed above helped researchers in the classification of other C2 domains existing in the different eukaryotic genomes by primary sequence alignments [1–3,17,41]. Two exceptions have been reported for the C2A domains of piccolo (long spliced form) and rabphilin 3A, where Ca^{2+} binding induces a significant conformational change and, in spite of conserving the key aspartate residues (Fig. 4A) they exhibit a very low affinity for Ca^{2+} [42–44]. Structural characterization of the Ca^{2+}-free structures have revealed that, in both cases, the disposition of the backbone and side chains of the aspartate residues adopt conformations incompatible with Ca^{2+} coordination [42–44]. The distance between the CBR1 and CBR3 is too wide in the case of piccolo, due to the structural reorganization induced by the extra nine amino acids alternatively spliced [42] and, however, too narrow in the case of rabphilin (Fig. 4B, C). However, how the structures that are incompatible with Ca^{2+} binding convert into Ca^{2+}-bound structures is something not well characterized yet but will be of crucial importance since they represent a specific regulation by Ca^{2+}-triggering for these domains. A recent report has shown an intermediate state for the Ca^{2+}-bound form of the C2A domain of rabphilin 3A, and suggest that the first Ca^{2+} occupying the CBR is the one in position Ca2, which initiates the reorganization of the side-chains of several aspartate residues, thus facilitating the entrance of the second Ca^{2+} in position Ca1 [45].

The final picture we get is that in many cases several members of the same family of C2-containing proteins (for example classical PKCs and synaptotagmins) bind Ca^{2+} with different affinities that are also modulated by the presence of membrane lipids. Taking into account that several of these proteins are located in the same cell membrane microdomains it is difficult to figure out their individual roles in a spatiotemporal dimension. A big effort has been performed to find the meaning of these subtle variations. For example, in the case of synaptotagmins and related proteins, a myriad of works have provided a better idea about the role of each isoform in concrete events of synaptic and endocrine exo- and endocytosis, revealing that this control is executed by a complex combination of calcium affinity, lipid affinity/selectivity, together with the relative abundance of the various proteins in this restricted microdomain (reviewed extensively in [41,46–51]).

3. The phospholipid binding site related to the calcium-binding region

As mentioned above, one major function of C2 domains is targeting membrane surfaces as a consequence of Ca^{2+}-binding. Extensive structural studies of C2 domains in complex with Ca^{2+} have enabled us to characterize the different calcium-binding modes. However, the impossibility to get crystal structures of these domains in complex with phospholipids has limited our knowledge to completely define the membrane docking mechanism for each domain. A vast number of biochemical and biophysical studies have demonstrated that C2 domains bind to their target membranes by using a combination of electrostatic and hydrophobic interactions. In addition, they display different phospholipid selectivity; for example, the C2 domains of classical PKCs and synaptotagmins bind to the anionic headgroup of phosphatidylserine [8,52–56] while the C2 domain of $cPLA_2$ binds to neutral phosphatidylcholine [24,57,58].

Pioneering structural characterization of the C2 domain of PKCα in complex with Ca^{2+} and 1,2-dicaproyl-*sn*-phosphatidylserine (DCPS) demonstrated two important facts: one was that the phosphoryl group of the phosphoserine completes the coordination sphere of Ca1 (Fig. 5A) [25,27], the other was that four more residues on the tip of the CBRs establish direct hydrogen bonds and hydrophobic interactions with the seryl moiety (N189), fatty acyl chains and fatty acyl *sn-1* and *−2* ester carbonyl groups (R249, R216 and T251) of the phospholipid, acting thus as a lid that covers the cup containing the calcium ions (Fig. 5A). These residues were shown to be important for PKCα activation and plasma membrane localization [59–61] and

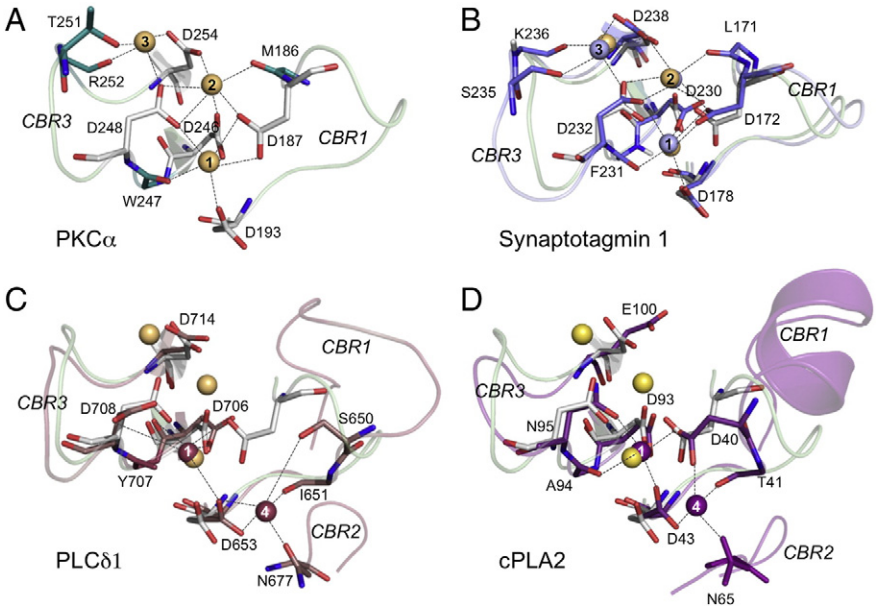

Fig. 3. Comparison of the calcium binding modes of C2 domains belonging to the PFAM/PKC-C2 family. (A) PKCα colored in green (PDB: 3GPE). (B) Synaptotagmin 1 is colored in light purple (PDB: 1BYN). (C) PLCδ is colored in raspberry (PDB: 1DJI). (D) $cPLA_2$ is colored in magenta (PDB: 1RLW). Calcium ions are represented as yellow spheres for PKCα and each corresponding color sphere for the rest of the domains. To facilitate the comparison and localization of the different calcium ions, we have overlapped each domain with that of PKCα. We have used the nomenclature stated by Rizo and Südhof [20] to name the Ca^{2+}-binding sites. The main aspartate residues involved in calcium coordination are represented by sticks in the color codes indicated above and are overlapped with those of PKCα (gray sticks), the main chain of residues that also contribute with oxygen atoms to calcium coordination are also shown as sticks. The different calcium binding regions have been labeled as CBR1-3.

also explained the reciprocal cooperativity observed for Ca^{2+} and phospholipids binding along the years [22,56,59,62,63] based on the phospholipid contribution to complete the Ca^{2+} coordination sphere. Very recently, the crystal structure of the C2B domain of synaptotagmin 1 in complex with Ca^{2+} and soluble phosphoserine has been solved (Fig. 5B) [64] and it shows different interactions than those described for PKCα. In this case, the carboxyl group of the phosphoserine moiety completes the coordination sphere of Ca1, and also interacts with the amide nitrogen of K366 (CBR3) (Fig. 5B). In addition, the amine group of phosphoserine establishes one hydrogen bond with D309 and, K366 forms a salt bridge with the phosphoserine phosphate group (Fig. 5B). These different results might have several explanations:

i) A soluble phosphoserine group was used to obtain the C2B domain crystal while a short acyl chain phospholipid (DCPS) was used for C2-PKCα. Taking into account that both carbonyl groups and acyl chains make important interactions with the protein, we cannot discard that the phosphoserine group adopted a conformation that

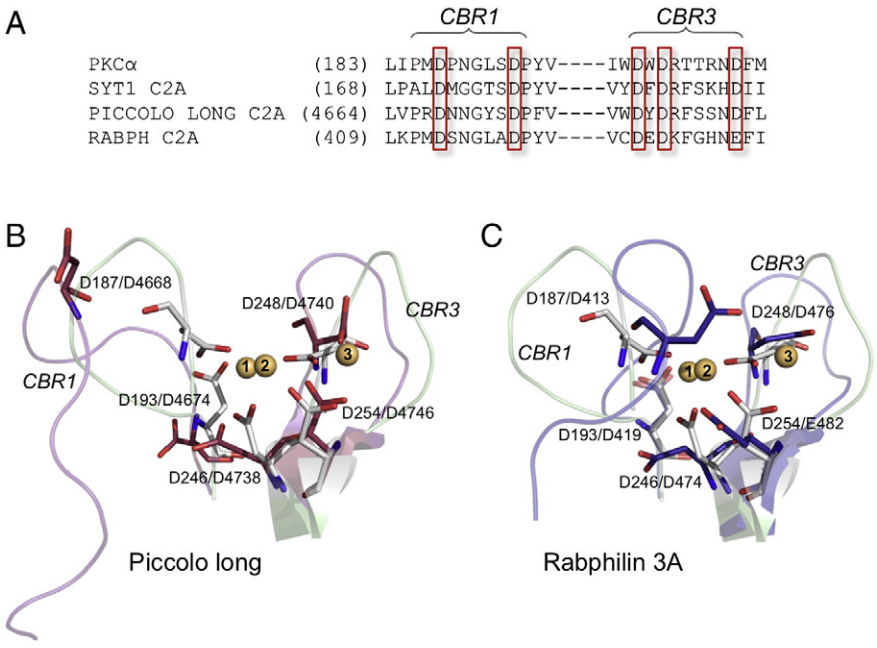

Fig. 4. Comparison of the calcium binding modes of C2 domains of piccolo and rabphilin 3A. (A) Primary sequence alignment of the CBR1 and CBR3 of PKCα, C2A-synaptotagmin 1, C2A-piccolo and C2A-rabphilin. The five key aspartate residues are included in the red rectangles. (B) Overlap of piccolo long colored in violet (PDB: 1RH8) with PKCα (green). (C) Overlap of rabphilin 3A colored in blue (PDB: 2CHD) with PKCα (green). Calcium ions are represented as yellow spheres for PKCα, the other two structures do not contain Ca^{2+}-bound, note how the side chains of the Asp residues are in conformations incompatible with Ca^{2+} coordination in both cases.

fits the needs of the calcium-binding region of the C2B domain in the absence of the key moieties like the glycerol and carbonyl groups.

ii) A 3D alignment of the C2-PKCα and C2B-synaptotagmin 1 domains (Fig. 5A–C) reveals that two of the key residues interacting with DCPS in C2-PKCα are not conserved in the C2B domain. This confers the top of the CBR of synaptotagmin a slightly lower electropositive potential (Fig. 6A) that concentrates only on one side of the CBR3 (mainly provided by K366). In addition, K332 in the CBR2 is a homolog to R216 in PKCα but does not participate in the lipid interaction due to the perpendicular conformation adopted by the phosphoserine in the C2B domain (Figs. 5 and 6A). Nonetheless, we cannot discard that a complete DCPS molecule might fit in this crevice by adopting another different conformation than the two described to date [25,64].

A very interesting observation rises when the 3D alignments and electrostatic surface potentials of C2A-synaptotagmin 1 and C2-PKCα are compared (Figs. 5C and 6A and B). The C2A domain of synaptotagmin 1 is a well-established phosphatidylserine binder [55,57,65], and conserves all the residues that contact DCPS in C2-PKCα with the exception of N189 that is substituted by glycine (G174) in C2A, rendering a very similar electropositive potential and suggesting that this CBR might fit a DCPS molecule in a very similar way to C2-PKCα. Note that R199 and K200 in the CBR2 could participate in hydrogen bonding the two-ester carbonyl groups of the phospholipid and the amide nitrogen of G174 could participate in hydrogen bonding with the carboxylate group of phosphoserine. Furthermore, if we compare the surface electrostatic potentials of both domains, the CBR1 and 3 regions at the top of the domain in PKCα are closer than in synaptotagmin 1 (Fig. 6A and B), and whether this is a lipid-dependent reorganization is something that will have to be further explored.

Substantial divergences with respect to C2-PKCα domain appear in the CBRs of PLCδ1, which does not conserve the arginine residues (R216 and R249) critical for DCPS interaction (Figs. 5C and 6B), nonetheless its electrostatic surface still provides an electropositive potential that together with N647 make possible the interaction with phosphatidylserine [66,67].

A completely different case is $cPLA_2$, whose CBR1 is longer than that of the others, forms a turn of α-helix (Fig. 3D) and interacts with neutral phospholipids like phosphatidylcholine. In addition, none of the residues involved in DCPS binding are conserved (Fig. 5C) and its electrostatic potential is hydrophobic, even after Ca^{2+}-binding (Fig. 6B), thus enabling the domain to deeply penetrate zwitterionic membranes [24,68,69]. Several studies have demonstrated that the top part of CBR1 and CBR3 are responsible for the different lipid selectivity. For example, grafting the short CBRs from the tip of C2-$cPLA_2$ onto the top of the C2A-synaptotagmin 1 domain confers the last one the phospholipid binding specificity of $cPLA_2$ [70]. Other works have demonstrated that these residues are also responsible for its specific subcellular localization [71,72].

The progress in the field reviewed here point to the idea that this family of C2 domains has evolved to bind biological membranes in a Ca^{2+}-dependent manner through two steps:

i) The development of a highly specific collection of aspartate residues located at the bottom of the CBR, which partially coordinate 2–3 calcium ions that neutralize the electronegative cleft. Moreover, they also serve as a bridge between the domain and the membrane since oxygen groups of the phospholipid serve to complete the Ca^{2+} coordination sphere.

ii) The domain additionally interacts with the membrane by residues located at the tip of the domain. The variability observed in these residues indicates that several combinations fulfill the criteria to bind to a specific phospholipid and suggest a more versatile adaptation of the domain to the lipids available at the membrane. Probably, these differences might affect the penetration and docking orientation in the membrane and in turn, modulate each protein activity.

4. The lysine-rich cluster: a conserved motif to specifically interact with phosphoinositides

Inositol phospholipids are involved in a broad variety of signaling pathways and also in the regulation of membrane traffic, the cytoskeleton, nuclear events and, permeability and transport of membrane channels [73]. PtdIns(4,5)P_2 is a member of the 7 stereospecific isoforms of the phosphoinositide family, which is phosphorylated at the 4′ and 5′ positions [74]. Early studies focused on its function as a precursor of intracellular messengers: diacylglycerol and IP_3 [75]. The discovery that the PtdIns(4,5)P_2 molecule itself and other bis- and tris-phosphorylated forms were also able to signal, expanded our view about new functions for this family of lipids [73,76,77]. In fact, inositol high polyphosphates and phosphoinositides have been described as targets for several C2

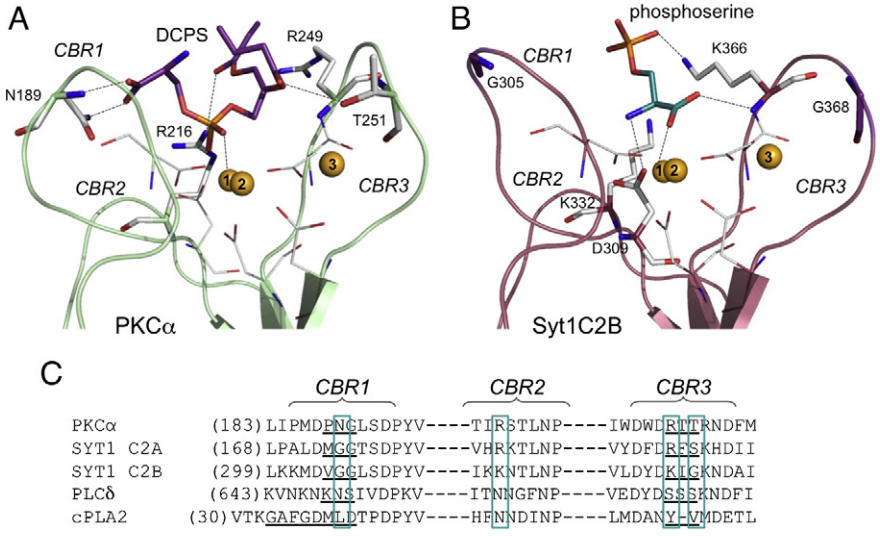

Fig. 5. C2 domains of PKCα and synaptotagmin 1 in complex with DCPS and phosphoserine, respectively. (A) CBR of the C2 domain of PKCα in complex with Ca^{2+} (yellow spheres) and DCPS (purple sticks). Side chains of the amino acids involved in lipid binding have been represented as gray sticks and the Asp residues involved in Ca^{2+} coordination are shown as gray lines at the bottom. (B) CBR of the C2B domain of synaptotagmin 1 in complex with Ca^{2+} (yellow spheres) and phosphoserine (blue sticks). Side chains of the residues involved in phosphoserine binding are represented as gray stick and the Asp residues as gray lines. (C) Primary sequence alignment of the CBR1-3; the residues involved in lipid binding are marked with a blue rectangle, the non-conserved residues when compared with PKCα are underlined in black.

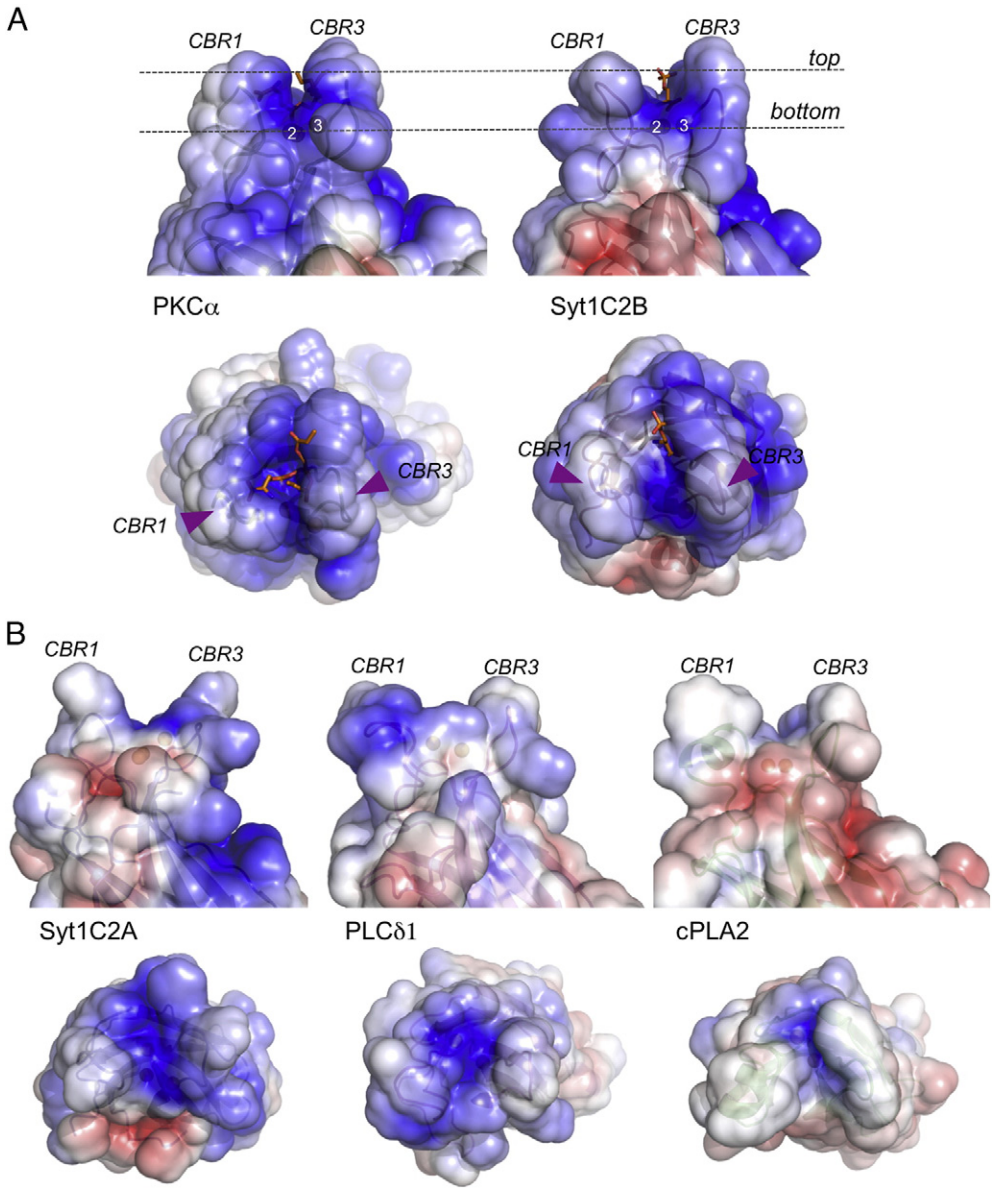

Fig. 6. Electrostatic surface representation of different C2 domains bound to Ca^{2+}. (A) The electrostatic surfaces of the C2 domain of PKCα and the C2B domain of synaptotagmin 1 were calculated in the presence of Ca^{2+}. (B) Electrostatic surfaces of the C2A domain of synaptotagmin 1, PLCδ1 and $cPLA_2$, calculated in the presence of Ca^{2+}. A side-view is represented on top. Below each domain there is a view from the top of the CBR. The potentials were calculated using the APBS plug-in from PyMOL and the surfaces were represented with the same program.

domains, including synaptotagmins, rabphilin 3A, DOC2 and classical PKCs among many others [78–82].

To date, the only X-ray structure of a C2 domain in complex with Ca^{2+}, phosphatidylserine and PtdIns(4,5)P_2 available is that of PKCα [21]. This structure demonstrates that the C2 domain of PKCα is able to interact with two distinct lipids: phosphatidylserine at the Ca^{2+}-binding region and PtdIns(4,5)P_2 through residues located in the β3 and β4 strands that have been named the *lysine-rich* or *polybasic cluster*, or the *β-groove*. In there, a collection of cationic (K197, K209 and K211) and aromatic (Y197, W245) residues establish direct interactions with the phosphate moieties of the inositol ring [21] (Fig. 7A). Indeed, substitutions of both aromatic and lysine residues in the full-length protein severely impair the ability of PKCα to translocate from the cytosol to the plasma membrane in a PtdIns(4,5)P_2-dependent manner [21,83–85].

Structure-based sequence alignment (VAST-MMDB) using as a template the 3D structure of the C2 domain of PKCα showed a high score for the C2 domains of synaptotagmins 1, 4, 7 and 13, rapbhilin 3A, PI3K-C2α, piccolo and RIM1, 2 [21,86,87]. Based on these results, a consensus PtdIns(4,5)P_2-binding sequence was proposed to include at least those C2 domains that conserve the six key residues [21] (Fig. 7B). Comparison of the primary sequences of this alignment showed that Y195, K197, K209 and N253 in PKCα are highly conserved in most of them (Fig. 7B). K211 was not well conserved in the C2A domains of some synaptotagmins and piccolo, which instead exhibit glutamate or valine residues. However, their polybasic clusters display many other lysine residues that still confer these motifs their electropositive potential, making difficult to discern if these residues are indispensable for PtdIns(4,5)P_2-binding specificity (Fig. 7B). A very recent report has demonstrated that substitution of this particular glutamate for lysine in the C2A domain of synaptotagmin 1 re-establishes the ability of the domain to interact with PtdIns(4,5)P_2, thus confirming the importance of this specific cluster in the phosphoinositide interaction [45].

Many evidences in the literature support this hypothesis, for example, a region including this motif has been known long ago since the early nineties. The injection of basic peptides corresponding to the homologous regions in the C2A and C2B domains of *Loligo pealei* synaptotagmin inhibited neurotransmitter release [88]. Later on, it

was demonstrated that this motif in the C2B domain of several synaptotagmins was responsible for interacting with inositol high polyphosphates (IP_4, IP_5 and IP_6) [89–91] and PtdIns(4,5)P_2 as well [78]. This region has also been proposed to mediate the interactions of synaptotagmin 1 with SNARE proteins syntaxin and SNAP-25 [92,93] and with AP-2, a protein involved in the clathrin-mediated endocytosis of synaptic vesicles on the plasma membrane [94–96].

Interestingly, IP_6 has been reported to act on the Ca^{2+}-binding site of the C2B domain of synaptotagmin 1 [97,98] and also to compete with the protein–protein interactions stated above [93,99], suggesting a regulatory role in vesicle exo- and endocytosis. The 3D structure (NMR) of the C2B domain of synaptotagmin 1 in complex with IP_6 indicates that it interacts with residues located at the lysine-rich cluster and the back of CBR3 [100]. One of the most important residues exhibiting intermolecular NOEs was K327, a homolog of K211 in PKCα (Fig. 7B and C) which is essential for the PtdIns(4,5)P_2-dependent membrane docking of the enzyme [21,84,85,101,102]. When the two structures are compared, the IP_6 molecule in the C2B domain of synaptotagmin 1 is displaced about 10 Å with respect to the theoretical PtdIns(4,5)P_2-binding site and closer to the CBR3 (Fig. 8A). Interestingly, the conformation of the CBR in this C2B domain structure is not compatible with Ca^{2+}-binding (Fig. 8B). Taking into account that the NMR experiments were performed in the presence of Ca^{2+} [100], it is possible that this particular IP_6 interaction with the domain impedes the Ca^{2+} entrance, mainly due to the distortion of the CBR1 and CBR3 backbones that make the side chains of D303, D365 and D371 adopt orientations incompatible with Ca^{2+} coordination (Fig. 8B). These results are in agreement with neurophysiological experiments that demonstrate that IP_6 inhibits the excitatory neurotransmission by impeding Ca^{2+} binding to the C2B domain of synaptotagmin 1 in hippocampal neurons [98] and also fit with the old observation that soluble inositol polyphosphates inhibit the binding of phosphoinositides to synaptotagmin in the absence of Ca^{2+} [78,97]. Therefore, these results pose a very attractive hypothesis of a non-competitive regulation executed by IP_6 to control the Ca^{2+}/PtdIns(4,5)P_2-dependent membrane fusion.

Additional works have demonstrated that PtdIns(4,5)P_2 interact with the conserved polybasic region of the C2B domain of synaptotagmin 1, both in the absence and presence of Ca^{2+} [64,81,103–105]. In fact, the most accepted hypothesis is that PtdIns(4,5)P_2 pre-binds to the C2B domain prior to the Ca^{2+} signal, and increases the speed of response of synaptotagmin to Ca^{2+} by enhancing its affinity for interaction [64,81,103,104,106]. Several reports have proved that the presence of PtdIns(4,5)P_2 in the target membrane is an absolute requirement for Ca^{2+}-triggered exocytosis and increases the rate of vesicle fusion by several folds [107,108]. This mutual cooperation has been also demonstrated for the membrane localization of PKCα both in vitro [84,109–112] and in living cells [85,101,102]. Together, these data show that the C2B domain of synaptotagmin 1 might be highly regulated by IP_6, PtdIns(4,5)P_2, Ca^{2+} and protein–protein interactions, all of these being concentrated in the polybasic cluster. Other C2 domain-containing protein that also binds PtdIns(4,5)P_2 is rabphilin 3A, a soluble double-C2 protein which is a Rab3/27 effector in neuronal cells [113,114] that participates in the re-priming of releasable vesicles at the plasma membrane [115]. Pioneering studies by Chung et al. [79] demonstrated that both C2A and C2B domains bind PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner and how phosphatidylserine cooperates to increase the affinity of the domain for binding PtdIns(4,5)P_2-containing membranes. The 3D structure of this domain in complex with a PtdIns(4,5)P_2 molecule has been solved recently by X-ray crystallography and demonstrates that the conserved residues located in the β3 and β4 strands are responsible for this interaction [45], being very similar to the model proposed for IP_3 binding in solution [44,116], and to the C2 domain of PKCα (Fig. 7A) [21].

DOC2 is another soluble tandem-C2 protein family that modulates secretory vesicle exocytosis in PC12 and chromaffin cells [117,118]. The polybasic sequence of the C2B domain has been found to be responsible for this function by interaction with the syntaxin-1A/SNAP-25 heterodimer [117]. Recent studies have involved DOC2 in several events at the synapsis, for example the regulation of Ca^{2+}-dependent and -independent spontaneous synaptic vesicle fusion [82,119] and asynchronous neurotransmitter release [120]. Groffen et al. demonstrated that the polybasic patch homologous to the C2B domain of rabphilin 3A, synaptotagmin 1 and C2 domain of PKCα is mediating the PtdIns(4,5)P_2 and the SNARE complex interaction, and both events can occur at the same time [82].

There are many other C2 domain-containing proteins like CAPS (Ca^{2+}-dependent secretion activator) [121] and PI3KC2α [122,123] that also play important functions at the pre-synaptic level and have been shown to interact with phosphoinositides. CAPS exhibits C2 and PH domains but how PtdIns(4,5)P_2 performs its function is not well characterized yet. Moreover, PI3KC2α has recently been involved in the spatiotemporal control of endocytosis by inducing the formation of PtdIns(3,4)P_2 [122].

The PtdIns(4,5)P_2–synaptotagmin relationship goes far beyond the synaptic neuron and very recently other members of the family, i.e. extended-synaptotagmin 1–3 have been shown to reside in the endoplasmic reticulum and participate in its tethering with the plasma membrane, PtdIns(4,5)P_2 being crucial to this event that occurs independently of Ca^{2+} for the isoforms 2 and 3, and is Ca^{2+}-dependent for isoform 1 [124].

The results reviewed here start to give us an idea about the dimensions of characterizing the PtdIns(4,5)P_2–C2 domain interaction for many biological processes, and it is obvious that achieving more 3D structures of different C2 domains in complex with phosphoinositides will provide us with invaluable information.

5. Conclusions and future perspectives

In this review we focused on describing the molecular mechanisms of Ca^{2+}- and negatively charged phospholipids binding to different C2 domains belonging to the PFAM/PKC-C2 family, especially those targeted to the membrane in a Ca^{2+}-dependent mode. We have also outlined the molecular mechanism of the PtdIns(4,5)P_2–C2 domain interaction and its consequences in several cellular events.

The vast structural, biochemical, biophysical and cellular information available to date, together with bioinformatic tools, have enabled us to define with high precision the Ca^{2+}-binding mechanism of C2 domains, which involves a collection of aspartate residues located at the bottom of what is defined as the calcium-binding region. These residues are pivotal to classify these domains as Ca^{2+}-dependent or -independent. However, why these domains exhibit different intrinsic Ca^{2+} affinities is not well understood. Several structural works have revealed that the 3D conformation of the CBRs of certain domains are not ready for Ca^{2+} binding, in spite of carrying the amino acids belonging to the consensus site, indicating that there might be more than one way to regulate the intrinsic Ca^{2+} affinity of these domains.

Crystal structures of C2 domains in complex with Ca^{2+} and negatively charged phospholipids, and the myriad of work from many laboratories indicate that the top of the CBR plays two important roles: i) to discriminate the target lipids at the membrane with certain variability in the sense that more than one amino acidic combination is compatible with membrane docking directed by the same lipid and ii) to stabilize the interaction of the lipid molecule with the domain with the consequent increase in the apparent Ca^{2+} affinity of the domain due to the “bridging effect” in which oxygen moieties from the lipid molecule complete the Ca^{2+} coordination sphere.

The finding that PtdIns(4,5)P_2 specifically interacts with the lysine-rich cluster, a different motif to the CBR, opens the possibility for this domain to be regulated by a dual-target mechanism. How these two targets combine in nature is something not clear yet and will probably depend on each particular cellular event. Whether the membrane composition has to fulfill the appropriate docking

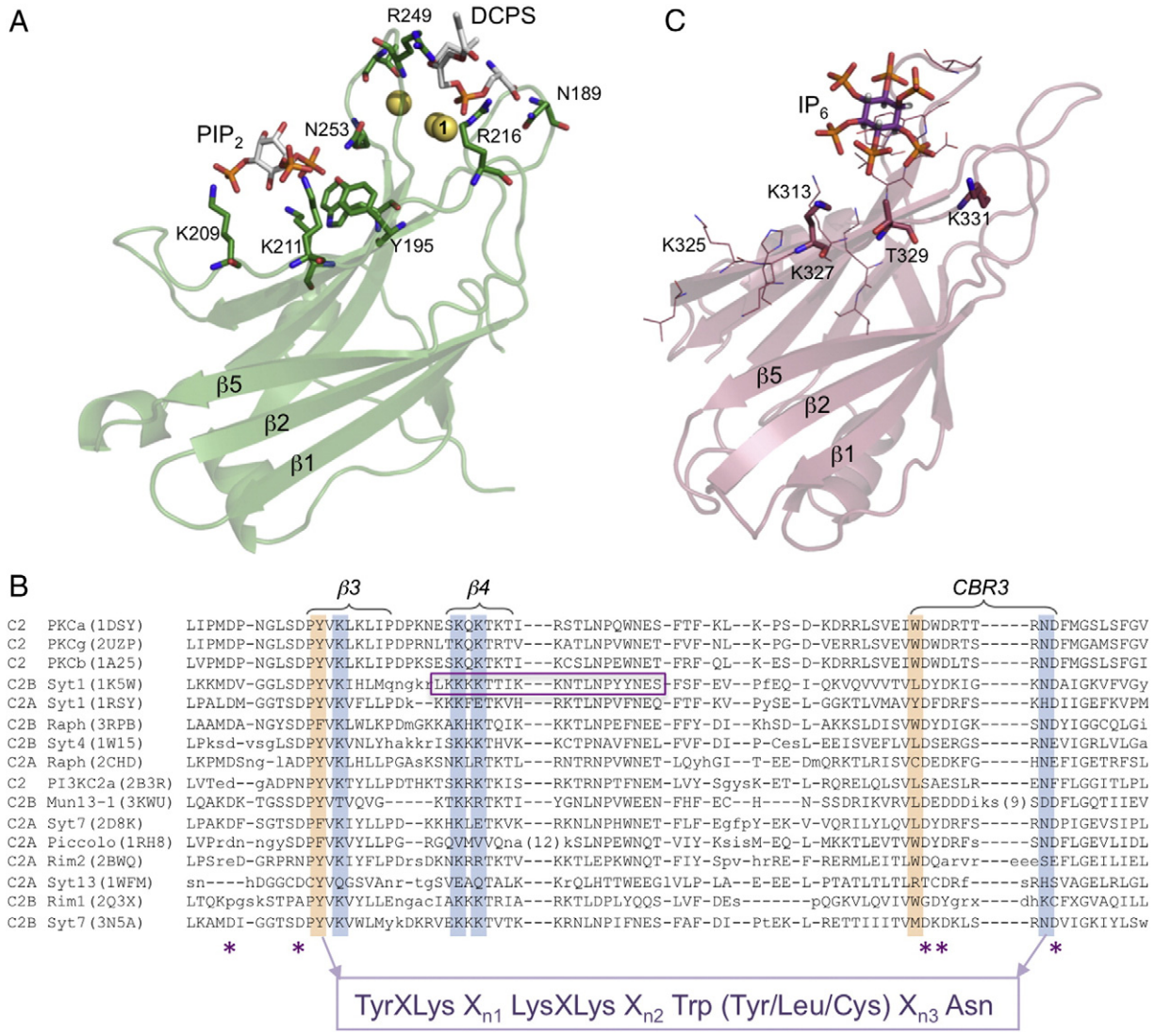

Fig. 7. Overall structures of the C2 domain of PKCα and C2B of synaptotagmin 1 in complex with phosphoinositides. (A) C2 domain of PKCα in complex with DCPS and PtdIns(4,5)P_2. Side chains of the residues involved in lipids interactions are represented as green sticks. The two phospholipids are shown in gray sticks and Ca^{2+} as yellow spheres. (B) Structure-based sequence alignment of the C2 domain of PKCα (1DSY), the primary sequences are classified in order from the highest to the lowest (19.2–10) Vast Score calculated by the VAST-MMDB Database (http://structure.ncbi.nlm.nih.gov/ Structure/VAST/vast.shtml). Protein identification and PDB codes are located on the left. Conserved Asp residues that participate in Ca^{2+} coordination are labeled with red stars at the bottom. The homologous residues involved in PtdIns(4,5)P_2-binding are shaded in blue for K197, K209, K211 and N253 and in yellow for Y195 and W245. The red box indicates the homologous residues of the 20 amino acid peptide demonstrated to be involved in high polyphosphate interactions for the C2B domain of some synaptotagmins. Blue box at the bottom represents the consensus sequence. (C) Cartoon representation of the C2Bsyt1-IP_6 complex structure. IP_6 is shown in stick representation, the three active residues included to determine the binary complex are represented by raspberry sticks (K327, T329 and K331), and the group of passive residues has been represented by lines.

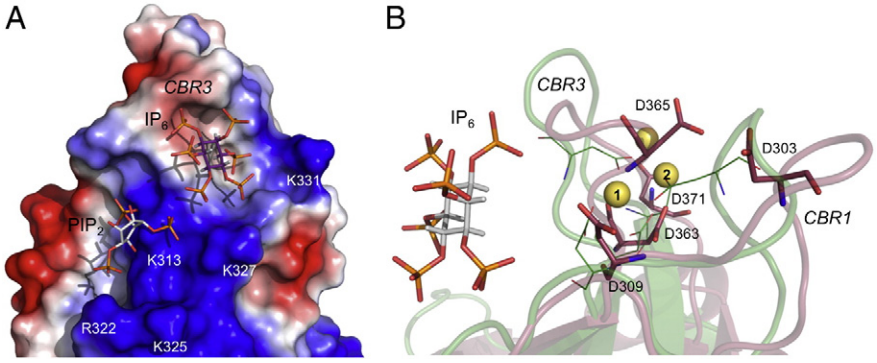

Fig. 8. (A) Surface representation of the C2Bsyt1–IP_6 complex structure, the PtdIns(4,5)P_2 molecule has been located by overlapping the structure of PKCα. (B) Overlap of the CBRs of the C2B domain of synaptotagmin 1 in complex with IP_6 (pink) and PKCα (green). Side chains of the residues involved in Ca^{2+} binding are represented as pink sticks for synaptotagmin and as green lines for PKCα, yellow spheres correspond to those bound to PKCα since synaptotagmin structure did not show any bound Ca^{2+}, note how the orientation of the aspartate side chains are not compatible with Ca^{2+} coordination.

conditions (two lipids in the same microdomain), or a single domain is able to respond to individual target lipids is something not well characterized, but the implications for the regulation of proteins that bear one or more C2 domains are enormous due to the multiple combinations that can be obtained if all of them are achievable.

This hypothesis opens a much more complicated picture of the structure–function paradigm since a single structure might influence different pathways depending on the signaling lipids generated at the membrane; not to mention if the protein carries more than one lipid-binding domain. The example of PKCα is very representative: it is a Ca^{2+}-dependent kinase containing two C1 and one C2 domains, the first interacts with diacylglycerol and the last interacts with phosphatidylserine and PtdIns(4,5)P_2 [76,125,126]. Classical experiments have demonstrated that it responds to a determined calcium concentration when it encounters phosphatidylserine and diacylglycerol at the plasma membrane in an organized spatiotemporal dynamic [127]; however, it will respond more rapidly and at lower calcium concentration if the membrane microdomains are enriched with PtdIns(4,5)P_2 [85].

Another classical example is the active zone where neurotransmitter release is organized at the presynaptic nerve terminal. In there, a myriad of proteins including a collection of C2 domain-containing proteins organize to dock and prime the synaptic vesicles for exocytosis. It is obvious that to get a precise spatiotemporal control of the process, each C2 domain needs to be regulated differently by itself and/or in collaboration with other domains/proteins around [128,129].

Whether an enzyme or synaptic protein will signal differently depending on the composition of the microdomains generated at the membrane in a particular cell event will have to be further explored. This possibility implies very important consequences in any of the fields of study since we will have to re-plan very classical experiments that now will include the correct lipid composition or re-interpret old experiments that only considered the basic assumptions available at that time.

Those are not easy hypotheses to test, but the huge development that the single-molecule and super-resolution microscopy techniques are experimenting, together with the design of new fluorescence probes specific to detect individual lipids are undoubtedly very important tools that this field will benefit from. Further exploration to obtain a wider number of 3D structures in complex with phospholipids, in combination with molecular dynamics approaches will also contribute to our understanding of their membrane-docking properties.

We also apologize for those works that have not been mentioned in this review due to the lack of space.

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